# Central role of Rho-kinase in the pathophysiology of allergic asthma

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# Chapter 11

## Protective effects of the inhaled Rho-kinase inhibitor Y-27632 on allergen-induced acute bronchoconstriction, airway hyperresponsiveness and inflammation

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## Abstract

Rho-kinase has recently emerged to be a potential target in allergic asthma. Using a guinea pig model of asthma, we have recently demonstrated that increased Rho-kinase activation contributes to the allergen-induced airway hyperresponsiveness (AHR) after the early (EAR) and late (LAR) asthmatic reaction, which is acutely reversed by inhalation of the specific Rho-kinase inhibitor Y-27632. In the present study, we investigated the protective effects of inhaled Y-27632 on the allergen-induced EAR and LAR, AHR to histamine after these reactions and airway inflammation.
Using permanently instrumented and unrestrained ovalbumin (OA)-sensitized guinea pigs, the OA-induced EAR and LAR were measured and histamine $PC_{100}$-values (provocation concentrations causing 100% increase in pleural pressure ($P_{pl}$)) were assessed 24 h before OA-challenge and at 6 and 24 h after the OA-challenge, *i.e.* after the EAR and LAR, respectively. Thirty minutes before and 8 h after OA-challenge, saline or Y-27632 (5 mM nebulizer concentration) was administered. After assessment of the last $PC_{100}$-value, bronchoalveolar lavage (BAL) was performed to determine the infiltration of inflammatory cells into the airways.
Inhalation of Y-27632 markedly reduced the acute allergen-induced bronchoconstriction, without a significant effect on the magnitudes of the total EAR and LAR expressed as area under the $P_{pl}$ time-response curve, however. In addition, Y-27632 considerably attenuated the AHR after the EAR and even normalized the AHR after the LAR. These effects could not be explained by a direct effect of Y-27632 on the increased histamine responsiveness at these time points, due to the short duration of the acute bronchoprotection of the inhaled dose of Rho-kinase inhibitor (<90 min). Moreover, Y-27632 reduced the number of total inflammatory cells, eosinophils, macrophages and neutrophils recovered from the BAL.
The results indicate that inhalation of Y-27632 protects against acute allergen-induced bronchoconstriction, development of AHR after the EAR and LAR, and airway inflammation. The anti-inflammatory effect of Y-27632 could be importantly involved in the protection against allergen-induced AHR.

## Introduction

Allergic asthma is an inflammatory airways disease characterized by allergen-induced early and late bronchial obstructive reactions, associated with infiltration and activation of inflammatory cells in the airways and the development of airway hyperresponsiveness (AHR) to a variety of stimuli, including contractile agonists such as histamine [1-3].

Agonist-induced smooth muscle contraction is regulated to an important extent by phosphorylation of the 20kDa myosin light chain ($MLC_{20}$) [4]. $MLC_{20}$ phosphorylation is initiated by an increase in intracellular $Ca^{2+}$-concentration ( $[Ca^{2+}]_i$ ) and subsequent formation of $Ca^{2+}$-calmodulin, resulting in activation of myosin light chain kinase

(MLCK). The extent of $MLC_{20}$ phosphorylation is determined by the balance between MLCK and myosin light chain phosphatase (MLCP) activities [5]. It has been established that contractile stimuli may not exert their effects exclusively by increasing $[Ca^{2+}]_i$, but also by increasing the sensitivity of the contractile apparatus to $Ca^{2+}$. The Rho/Rho-kinase pathway has emerged to be a key regulator of this $Ca^{2+}$-sensitization [5-7]. Activated Rho-kinase inactivates the myosin binding subunit of MLCP by phosphorylation, thereby interfering with the equilibrium of MLCK and MLCP activities. This leads to an augmentation of $MLC_{20}$ phosphorylation and hence an elevation of contraction at an established $[Ca^{2+}]_i$ [6,8].

Only recently, Rho-kinase has come forward to be a potential target in airways diseases, including allergic asthma [7]. It has been demonstrated that Rho/Rho-kinase-mediated $Ca^{2+}$-sensitization is enhanced in acetylcholine-induced contraction of bronchial smooth muscle obtained from repeatedly allergen-challenged rats [9]. Moreover, we have previously demonstrated that active allergic sensitization, without subsequent allergen exposure, was sufficient to increase the role of Rho-kinase in guinea pig tracheal smooth muscle contraction *ex vivo* and airway responsiveness *in vivo* [10][Chapter 8]. In accordance, passive sensitization-induced nonspecific ASM hyperresponsiveness and specific allergen responsiveness in these preparations were found to be dependent on Rho-kinase as well [11][Chapter 9]. The enhanced contribution of Rho-kinase to airway responsiveness could involve increased expression of RhoA, as protein levels of this upstream activator of Rho-kinase have been reported elevated both after allergic sensitization in guinea pigs [10][Chapter 8] and after repeated allergen challenge in rats [9] and mice [12]. Clearly, although Rho-kinase is involved to some extent in the regulation of airway smooth muscle tone under control conditions [13,14][Chapters 2 & 4], an increased contribution of Rho-kinase to airway responsiveness is evident under pathophysiological conditions. Further supporting such a pathophysiologically primed role, we have very recently demonstrated that in addition to a bronchoprotective effect under basal conditions, inhalation of the Rho-kinase inhibitor Y-27632 acutely reverses allergen challenge-induced AHR after the early (EAR) and late (LAR) asthmatic reaction in permanently instrumented, unanaesthetized and unrestrained guinea pigs [15][Chapter 10]. Moreover, it was found that an increased Rho-kinase activity is involved in the development of the allergen-induced AHR, as indicated by an enhanced effectiveness of Y-27632 to reverse the increased airway responsiveness to histamine and $PGF_{2\alpha}$, both after the EAR and the LAR [15][Chapter 10].

Thus far, investigations have mainly been focused on the acute effects of Rho-kinase inhibitors on airway (hyper)responsiveness. The present study was designed to assess the putative protective effects of pretreatment with inhaled Y-27632 on the development of AHR after the allergen-induced EAR and LAR, in relation to its effects on the bronchial obstructive reactions and airway inflammation.

## Methods

*Animals*

Outbred specified pathogen-free male Dunkin Hartley guinea pigs (Harlan, Heathfield, U.K.), weighing 600-700 g, were used in this study. The animals were actively IgE-sensitized to ovalbumin (OA) as described previously [16,17]. In short, 0.5 ml of an allergen solution containing 100 μg/ml OA and 100 mg/ml $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the legs, lumbar regions and the neck. The animals were operated on 2 weeks after sensitization and used experimentally in weeks 4 to 8 after sensitization. The animals were group-housed in individual cages in climate-controlled animal quarters and given water and food *ad libitum*, while a 12-h on/12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

*Measurement of airway function*

Airway function was assessed in conscious, permanently instrumented, unrestrained guinea pigs, by on-line measurement of pleural pressure ($P_{pl}$) as described previously [16,18]. In short, a small saline-filled balloon-catheter was surgically implanted inside the thoracic cavity. The free end of the catheter was driven subcutaneously to the neck of the animal, where it was exposed and attached permanently. Via an external saline-filled canula the pleural balloon-catheter was connected to a pressure transducer (Ohmeda DTX, SpectraMed, Bilthoven, the Netherlands), a signal conditioner, and an on-line computer system, enabling continuous measurement of $P_{pl}$ changes (in cm $H_2O$). We have previously demonstrated that changes in $P_{pl}$ are linearly correlated with changes in airway resistance and hence can be used as a sensitive index for bronchoconstriction [18].

*Provocation procedures*

Provocations with OA and histamine as well as administration of Y-27632 were performed by inhalation of aerosolized solutions. Aerosols were produced by a DeVilbiss nebulizer (type 646; DeVilbiss, Somerset, PA, USA), driven by an airflow of 8 l/min and resulting in an output of 0.33 ml/min. Provocations were carried out in a perspex cage (internal volume of 9 l) in which the guinea pigs could move freely [16,18]. Before the start of the experiment, the animals were habituated to the experimental conditions on three sequential days at least one week after surgery, when preoperative weight had been restored. On the first day, the animals were placed in the provocation cage unconnected to the pressure transducer. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each exposure lasting 3 min and separated by a 7-min interval. The next day, this procedure was repeated with the animals connected to the measurement system. Finally, a histamine-challenge, as described below, for habituation was performed on the third day.

On the experimental days, following the habituation procedure, histamine and OA provocations were performed. All provocations were preceded by an adaptation period

of at least 30 min, followed by two consecutive control provocations with saline. Baseline $P_{pl}$ was calculated by averaging the $P_{pl}$ of the last 20 min of the adaptation period.
To assess the airway reactivity to histamine, provocations were performed with an initial 25 µg/ml histamine solution in saline, followed by increasing dosage steps of 25 µg/ml. Histamine provocations lasted 3 min, separated by 7 min intervals. Animals were challenged until $P_{pl}$ was increased by more than 100 % above baseline for at least 3 consecutive minutes. $P_{pl}$ returned to baseline value within 15 min after the last provocation. The provocation concentration causing a 100 % increase of $P_{pl}$ ($PC_{100}$-value) was derived by linear intrapolation of the concentration-$P_{pl}$ curve and was used as a measure for airway reactivity toward the agonist. OA-provocations were performed by inhalation of increasing aerosol concentrations of 0.5 and 1.0 mg/ml OA in saline for 3 min, separated by 7 min intervals. In the saline-treated control animals allergen inhalations were discontinued when an increase in $P_{pl}$ of more than 100 % was observed, and the nebulized dose of OA was determined. Identical doses of OA were used in weight and age-matched animals treated with Y-27632, as indicated below. Using these conditions, no anti-histamines were necessary to prevent anaphylactic shock after allergen provocation.

*Provocation protocol*
Histamine $PC_{100}$-values were assessed 24 h before OA-challenge and at 6 h and 24 h after the OA-challenge, *i.e.* after the early (EAR) and late (LAR) asthmatic reaction, respectively [16,19]. Thirty minutes before and 8 h after OA-challenge, saline or Y-27632 (5 mM) was nebulized during 5 min.
For the quantitative assessment of the EAR (between 0 h and 5 h after allergen provocation) and LAR (between 8 h and 23 h after allergen provocations), airway function was continuously measured during the whole procedure. Between the measurements of histamine $PC_{100}$-values at 6 h and 24 h, the animals were placed in their home cage (0.16 $m^2$), in which water and food were freely accessible and where they could move around freely. During this transfer the animals remained connected to the measurement system.
In a separate set of experiments the duration of the acute bronchoprotective effect of inhaled Y-27632 was examined. At 15 min after the assessment of basal histamine $PC_{100}$, Y-27632 was aerosolized (5 mM nebulizer concentration) for 5 min after which histamine $PC_{100}$-values were reassessed at 0.3, 1.5, 3, 6 and 24 h.

*Bronchoalveolar lavage*
One hour after assessment of the last $PC_{100}$-value, animals were anaesthetised with 20 mg/ml Brietal-sodium, 35 mg/kg ketamine hydrochloride and 6 mg/kg Rompun i.p., which ensured a fast, deep anaesthesia. Using a tracheal canula, the lungs were lavaged gently using 5 ml of sterile saline at 37 °C, followed by three subsequent aliquots of 8 ml saline. The recovered samples were placed on ice, and centrifuged at 200 g for 10 min at 4 °C. The combined pellets were resuspended to a final volume of 1.0 ml in phosphate-buffered saline (PBS) and total cell numbers were counted using a coulter counter

(Beckman). For cytological examination, cytospin-preparations were stained with May-Grünwald and Giemsa stain. A cell differentiation was performed by counting at least 400 cells in duplicate.

*Data analysis*
The magnitude of the EAR after allergen provocation was expressed as the initial maximal increase in $P_{pl}$, which is mainly attributed to the release of mast cell-derived histamine [20,21], as well as the area under the $P_{pl}$ time-response curve (AUC) between 0 and 5 h after provocation, calculated by trapezoid integration over discrete (5 min) time-periods. The magnitude of the LAR was expressed as the AUC between 8 and 23 h after provocation. All data represent means ± s.e. mean from *n* separate experiments. Statistical significance of differences was evaluated using an unpaired two-tailed Student's t-test, a repeated measures one way analysis of variance (ANOVA) followed by a Holm-Sidak post-test or a one way ANOVA on ranks followed by a Dunn's post-test as appropriate. Significance was accepted when $P<0.05$.

*Chemicals*
Ovalbumin (grade III), aluminium hydroxide, histamine dihydrochloride and May-Grünwald and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632) was obtained from Tocris Cookson Ltd. (Bristol, U.K.). Ketamine hydrochloride was from Parke-Davis (Barcelona, Spain) and Rompun (2-(2.6-xylidino)-5.6-dihydro-4H-1.3-thiazine-hydrochloride, methylparaben) from Bayer (Leverkusen, Germany). All other chemicals used were of analytical grade.

## Results

In Figure 1a examples of online-recordings of $P_{pl}$ in guinea pigs after OA-challenge and pretreatment with saline or Y-27632 are shown. Overall analysis of these recordings revealed that Y-27632-treated animals displayed a marked reduction of the initial peak response in $P_{pl}$ (Figure 1b), which is mainly attributed to the release of histamine in the airways. However, the AUC of the EAR was not significantly affected (2562 ± 435 % x 5 min in saline-treated animals and 2579 ± 811 % x 5 min in Y-27632-treated animals). Also, no effects of Y-27632-inhalation on AUC of the LAR were observed (9663 ± 1261 % x 5 min and 10224 ± 2411 % x 5 min, respectively).

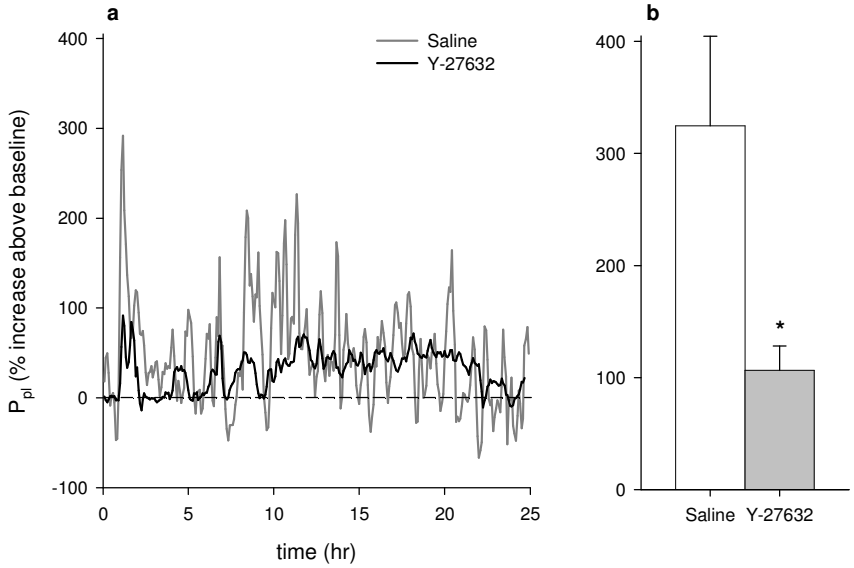


**Figure 1.** (a) Representative on-line recordings of $P_{pl}$ in sensitized, conscious and unrestrained guinea pigs after allergen challenge at t= 0 h, the animals being treated with inhaled saline (grey line) or Y-27632 (5 mM nebulizer concentration; black line) at 30 min before and 8 hr after the challenge. (b) Effects of pretreatment with inhaled saline or Y-27632 on the initial peak rise in response to allergen challenge. Results are presented as percentage increase in $P_{pl}$ above baseline. Data represent means ± s.e.mean of 4 to 5 animals. *P<0.05 compared to saline-treated animals.

As shown in Figure 2, OA-challenge induced a significant AHR in response to histamine after both the EAR and the LAR in the saline-treated control animals, as indicated by the reduced $PC_{100}$-values after these reactions. As compared to the saline-treated animals, pretreatment with inhaled Y-27632 resulted in a marked reduction of the AHR after the EAR, as indicated by a significantly smaller decrease of the absolute $PC_{100}$-value (Figure 2a) and a significantly reduced ratio of $PC_{100}$ pre/post challenge (Figure 2b). Notably, in the Y-27632-treated animals the AHR after the LAR was fully normalized to prechallenge conditions.

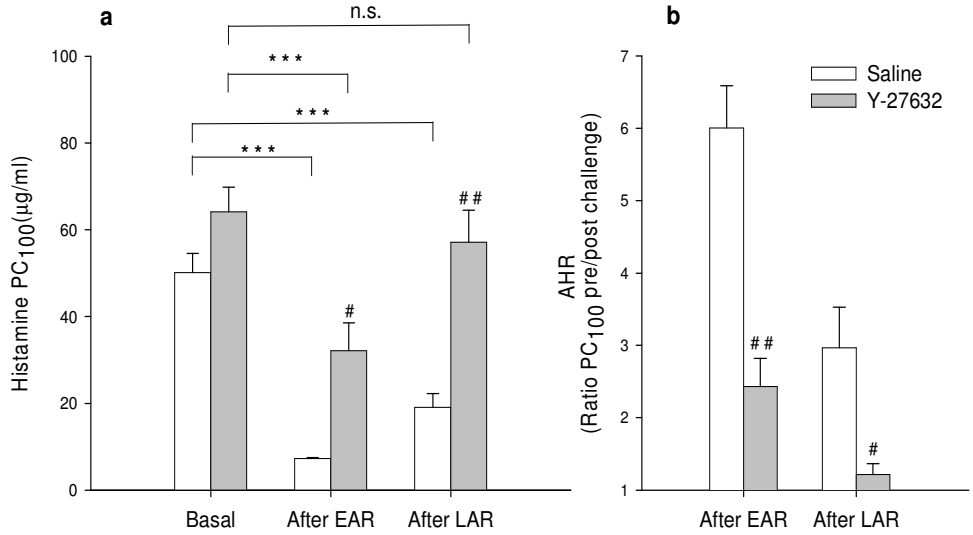


**Figure 2.** The protective effects of Y-27632 inhalation on the development of allergen-induced airway hyperresponsiveness after the early (EAR) and late (LAR) asthmatic reaction. (a) Effects of saline (white bars) and Y-27632 (5 mM nebulizer concentration; grey bars) inhalations, performed 30 min before and 8 h after allergen challenge on histamine $PC_{100}$-values after the EAR (measured at 6 h after allergen challenge) and LAR (24 h after the challenge). Basal represents the $PC_{100}$-values as assessed before saline or Y-27632 inhalation. (b) The degree of airway hyperresponsiveness (AHR) after the EAR and the LAR in saline (white bars)- and Y-27632 (grey bars)-treated animals, expressed as the ratio $PC_{100}$ pre/post challenge. A ratio of 1 signifies normo-responsiveness. Data represent means ± s.e.mean of 6 to 7 animals. *P<0.05, **P<0.01, ***P<0.001 compared to basal. $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared to saline.

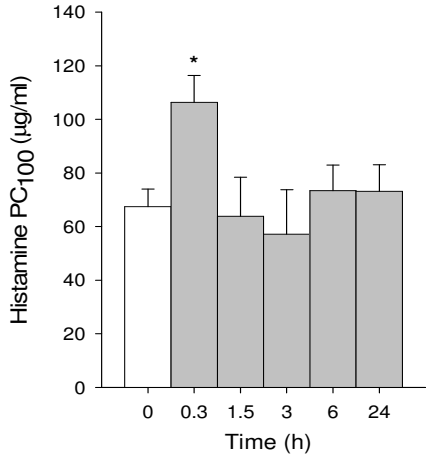


**Figure 3.** Time-course of the acute bronchoprotective effect of inhaled Y-27632 on histamine-induced bronchoconstriction. Data represent means ± s.e.mean of 3 to 4 animals. *P<0.05 compared to time-point 0 (*i.e.* before Y-27632 inhalation).

In separate experiments, it was established that the bronchoprotective effect of the Y-27632 inhalation on histamine-induced airways obstruction is abolished within 90 min (Figure 3), indicating that the effects of Y-27632 inhalation on the AHR after the EAR (6h after challenge) and LAR (24h after challenge) are not due to an acute bronchoprotection.

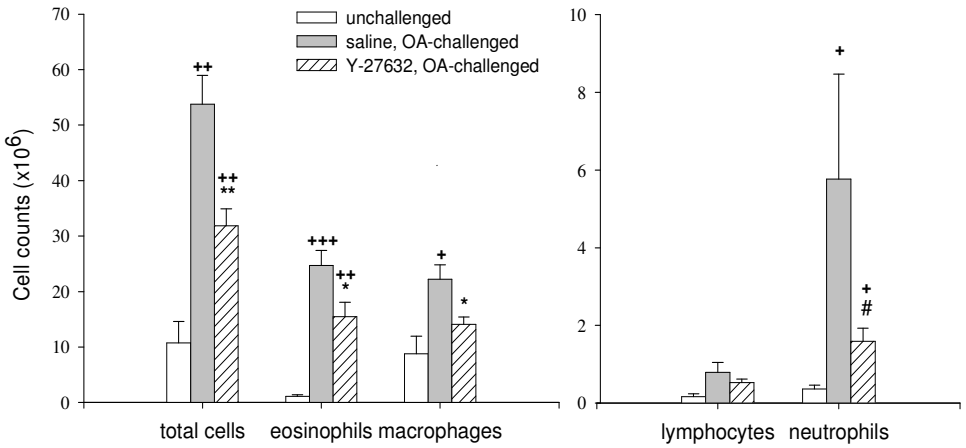


**Figure 4.** The effects of saline (grey bars) and Y-27632 (hatched bars) inhalations performed 30 min before and 8 h after allergen challenge on the inflammatory cell profile in the bronchoalveolar lavage obtained at 25 h after the allergen challenge as compared to unchallenged conditions. Data represent means ± s.e.mean of 4 to 7 animals. *P<0.05, **P<0.01, # P=0.07 compared to saline-treated, OA-challenged; +P<0.05, ++P<0.01, +++P<0.001 compared to unchallenged.

Since inhibition of airway inflammation could be involved, BAL was performed one h after assessment of the last $PC_{100}$-value to determine infiltration of inflammatory cells in the airways. In the saline-treated controls, OA-challenge induced significant increases in total inflammatory cell number, eosinophils, macrophages and neutrophils, which was significantly inhibited by treatment with inhaled Y-27632 (Figure 4).

## Discussion

In the present study, we have demonstrated that inhalation of the Rho-kinase inhibitor Y-27632 at 30 min before and 8 h after allergen challenge effectively prevents the development of AHR to histamine both after the EAR (measured at 6h after allergen challenge) and the LAR (measured at 24 h after allergen challenge). Since it was established that the acute bronchoprotective effect of the inhaled dose Y-27632 against histamine disappears within 90 min, the observed effects cannot be explained by a direct inhibitory effect of the Rho-kinase inhibitor on the histamine-induced airway constriction after the EAR and LAR. Rather, the observed effects of Y-27632 inhalation may at least in part be explained by an inhibition of infiltration of inflammatory cells into the airways, as the numbers of total inflammatory cells, eosinophils, neutrophils and macrophages were reduced in Y-27632-treated animals.

Previous studies have indicated a relationship between infiltration and activation of particularly eosinophils and AHR. Thus, in BAL fluid obtained from asthmatic patients, the number of eosinophils is increased and inversely correlates with the histamine $PC_{20}$ in these patients [22]. In addition, in biopsy studies a positive correlation was found between (activated) $EG2^+$ eosinophils and AHR [23]. Also, in mild asthmatics, a significant relationship between the amount of eosinophil-derived major basic protein (MBP) and AHR to histamine was found [22]. Accordingly, in our guinea pig model of allergic asthma a significant correlation was found between eosinophil peroxidase in BAL fluid and AHR at 6 h after allergen challenge [24]. Although the role of the eosinophil in allergic asthma has been challenged, there is compelling evidence for the involvement of eosinophils in various processes that may induce AHR [25]. As a source of basic granule proteins, growth factors, lipid mediators, pro-inflammatory cytokines and chemokines, eosinophils have for instance been associated with the induction of epithelial damage, $M_2$ autoreceptor dysfunction on cholinergic nerves, mucus hypersecretion, deficiency of cNOS-derived NO, and airway remodeling [25-30].

In agreement with our observations, it was recently found that intranasal application of Y-27632 prior to repeated allergen challenge reduces pulmonary eosinophilia and AHR to intravenously applied methacholine in anaesthetized mice [31]. The effects of Y-27632 on eosinophilia are consistent with *in vitro* findings demonstrating a crucial role for RhoA/Rho-kinase signaling in the chemotaxis of eosinophils in response to the chemokine eotaxin [32] and in the migration of eosinophils through endothelial barriers [33].

In addition to its effect on airway eosinophilia, our current data indicate that Y-27632 inhalation reduces the number of infiltrated macrophages and neutrophils as well. Currently, the role of neutrophils and macrophages in the development of AHR is incompletely understood. However, neutrophils have been implicated in the development of allergen challenge-induced acute AHR [34]. In addition, *in vitro* experiments have shown that supernatants from activated neutrophils induce

hyperresponsiveness of human bronchial smooth muscle preparations in response to electrical field stimulation, with the site of action presumably being prejunctional on the parasympathetic nerve [35]. In a murine model of allergic asthma, it was indicated that accumulation of neutrophils and activated macrophages in the airway wall was at least in part related to AHR [36].

As for eosinophils, reduced infiltration of neutrophils and macrophages could be explained by effects of RhoA and Rho-kinase on the migration of these cells [37-41]. Some of these effects may be linked to effects on endothelial barrier integrity. Actomyosin-mediated endothelial cell contraction is a key process in inflammatory cell-induced reduction in endothelial barrier function, which is required for tissue infiltration by inflammatory cells. Reduction of myosin phosphorylation in HUVEC endothelial cells by Rho-kinase or MLCK inhibition preserves endothelial barrier integrity and prevents inflammatory cell transit [39]. Though studied *in vitro*, these effects could be important *in vivo* as well, as it was demonstrated that in the lungs of a mouse model of acute lung injury induced by endotoxin, Y-27632 attenuated both oedema and migration of neutrophils, likely by modulating endothelial cell contraction induced by inflammatory mediators, rather than through an effect on neutrophil sequestration [42]. Although the infiltration of inflammatory cells into the airways appears to be Rho/Rho-kinase dependent, the specific link to this pathway is complex and requires further study.

In the present study, we also demonstrated that inhalation of Y-27632 significantly reduces the acute allergen-induced bronchoconstriction as indicated by the considerable attenuation of the initial peak rise in $P_{pl}$, which is believed to be mediated by histamine [20,43,44]. On the one hand, this might be explained by the bronchoprotective effect of Y-27632 inhalation on histamine responsiveness, as has also been observed previously [15]. An additional mechanism by which Rho-kinase inhibition could reduce this peak rise and perhaps even modulate airway inflammation and subsequent development of AHR might be through attenuating the release of mast cell-derived histamine. *In vitro*, using rat peritoneal mast cells, a role for Rho-kinase in regulating histamine-release has been reported. [45]. Several lines of evidence indicate that histamine may be involved in the development of allergen-induced airway inflammation and AHR. Thus, it has been described that histamine is capable of inducing the expression of adhesion molecules on endothelial cells [46] and potently increases vascular permeability. Accordingly, previous work from our laboratory demonstrated that inhalation of the histamine $H_1$-receptor antagonist mepyramine strongly reduced the allergen-induced AHR, both after the EAR and the LAR, and airway inflammation in the same guinea pig model as used in the present study [21].

The inhibition of the initial allergen-induced constriction by Y-27632 was not associated with an inhibition of the magnitude of the EAR when expressed as the AUC. This could be explained by the relatively short duration of the effect of the Rho-kinase inhibitor and the small contribution of the $P_{pl}$ peak to the entire AUC. Nevertheless, this effect may be of importance as it represents the largest decrease in lung function after the challenge.

Similarly, a short duration of action could explain why a significant effect of Y-27632 on the AUC of the LAR was absent. Remarkably, however, the infiltration of inflammatory cells into the airways was markedly inhibited, which may suggest a dissociation between airway inflammation and the LAR.

Collectively, our previous [15] and current findings indicate a pivotal role for Rho-kinase in regulating the development of the AHR in response to allergen challenge. Thus, in addition to the acute reversal of AHR after the EAR and the LAR by inhalation of Y-27632 [15], the present study indicates that pretreatment with the inhaled Rho-kinase inhibitor protects against the development of AHR after these reactions. This effect is associated with attenuation of the acute allergen-induced bronchoconstriction and airway inflammation. The anti-inflammatory effect of Y-27632 could be importantly involved in the protection against allergen-induced AHR. Altogether, these findings support a pharmacotherapeutical interest for Rho-kinase inhibitors in the treatment of allergic asthma.

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